



The phosphodiesterase 4 inhibitor AA6216 suppresses activity of fibrosis-specific macrophages

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ABSTRACT

Background: Idiopathic pulmonary fibrosis (IPF) is a form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, with a poor prognosis. We previously showed the antifibrotic effects of a novel phosphodiesterase 4 (PDE4) inhibitor, AA6216. In this study, we examined the effect of AA6216 on the pulmonary accumulation of segregated-nucleus-containing atypical monocytes (SatMs), which produce tumor necrosis factor (TNF)- α and are involved in murine lung fibrosis.

Methods: Mice were treated with bleomycin intratracheally at day 0 and either 10 mg/kg AA6216, 100 mg/kg nintedanib, or vehicle orally once daily from day 0 to 8. On day 9, we isolated the bronchoalveolar lavage fluid and analyzed the SatM ratio. In addition, we evaluated the effect of AA6216 on TNF- α production from SatMs isolated from murine bone marrow.

Results: AA6216, and not the antifibrotic agent nintedanib, significantly suppressed the pulmonary accumulation of SatMs (AA6216: $68.3 \pm 5.4\%$, Nintedanib: $129.8 \pm 19.7\%$). Furthermore, AA6216 dose-dependently inhibited the production of TNF- α by SatMs.

Conclusions: AA6216 suppresses pathogenic SatMs in the lung, which contributes to its antifibrotic effects.

1. Background

Idiopathic pulmonary fibrosis (IPF) is a form of chronic fibrosing interstitial pneumonia of unknown etiology. IPF is progressive, and the prognosis is generally poor. Two anti-fibrotic agents, nintedanib and pirfenidone, are available for the treatment of patients with IPF [1–3], but additional drugs with greater efficacy and fewer side effects are needed.

The pathogenic mechanisms leading to excessive collagen deposits in fibrotic lesions are poorly understood. However, cytokines, such as tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- β , are known to have important roles in the pathological process [4–6]. In fibrosis, monocytes and macrophages also play crucial roles. Recently, segregated-nucleus-containing atypical monocytes (SatMs) were identified as important contributors to the pathology of a bleomycin-induced mouse model of pulmonary fibrosis [7]. The authors discovered that SatMs, which are characterized by specific markers (Ceacam1⁺ Msr1⁺ Ly6C⁺ F4/80⁺ Mac1⁺), were critical for the development of lung fibrosis

in these mice [7]. The SatMs were found to accumulate in the fibrotic area at the initiation of fibrosis and to produce TNF- α , which leads to fibroblast activation [7].

Phosphodiesterase 4 (PDE4) is a cAMP-specific PDE that is expressed almost exclusively in inflammatory cells and that is a prime target of antifibrotic therapies. It directly regulates inflammatory responses, including the activation of macrophages [8,9]. We previously demonstrated the antifibrotic effects of a novel PDE4 inhibitor, AA6216 ((S)-5-chloro-2-(2-methylpiperazin-1-yl)-7-(thiazol-2-yl)-4-(trifluoromethoxy)benzo[d]oxazole), on non-clinical IPF-related models and cells cultured from patients with IPF [10]. Interestingly, we demonstrated that AA6216 inhibited TNF- α production by alveolar macrophages from IPF patients and reduced pulmonary fibrosis in a bleomycin-induced mouse model.

In this study, to clarify the effects of PDE4 inhibitors on SatMs, we investigated the impact of AA6216 on SatM accumulation in the murine lung and the production of TNF- α by SatMs.

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2. Materials and methods

2.1. Materials

AA6216 hydrochloride was synthesized by Meiji Seika Pharma Co., Ltd. Apremilast and roflumilast were purchased from MedChemExpress (Princeton, NJ, USA) and Toronto Research Chemicals (Toronto, Canada), respectively. Nintedanib was purchased from LC Laboratories (Woburn, MA, USA). A mouse TNF- α ELISA kit was purchased from R & D Systems (Minneapolis, MN, USA).

2.2. Animals

Seven-week-old specific pathogen-free female C57/BL6J mice were used for all experiments. All mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were housed in cages on a 12 h light-dark schedule with food and water *ad libitum*. All experiments were approved by the Animal Care and Use Committee of Kindai University Faculty of Medicine and the Animal Care and Use Committee of Meiji Seika Pharma Pharmaceutical Research Center.

2.3. Bleomycin model

Mice were anesthetized with 3% isoflurane delivered in a box. Under anesthesia, 1.5 mg/ml bleomycin hydrochloride (Nihon Kayaku, Tokyo, Japan) in 40 μ L of saline was administered intratracheally via a *trans*-oral route (day 0). Vehicle, 10 mg/kg AA6216, or 100 mg/kg nintedanib, dissolved in 0.5% methylcellulose, were administered orally once daily from day 0 to day 8. The dose of AA6216 was determined based on the achievement of high lung tissue concentrations and anti-fibrotic effects at this dose in our previous study [10]. The sample sizes were: vehicle, n = 20; AA6216, n = 20; and nintedanib, n = 10. Mice were sacrificed by exsanguination under anesthesia with 3% isoflurane on day 9. After the thoracic cavity was opened, bronchoalveolar lavage fluid (BALF) was obtained by bronchoalveolar lavage following 3 consecutive washes of the lungs with 0.8 mL PBS flushed through a tracheal cannula. BALF was then centrifuged at 300 g for 10 min at 4 $^{\circ}$ C. The cell pellets were resuspended in PBS and used for flow cytometric analyses.

2.4. Flow cytometry and cell sorting

The following antibodies were purchased from BioLegend: anti-F4/80 (BM8); anti-Mac1 (M1/70); anti-Ly6C (AL-21); and anti-Ceacam1 (Mab-CC1). Anti-Msr1 (LS-c43466) was obtained from LSBio. Cells were washed in ice-cold fluorescence activated cell sorting (FACS) buffer (2% FBS, 1 mM EDTA, 25 mM HEPES in HBSS), then incubated with each antibody for 30 min and washed twice with FACS buffer. Flow cytometric analysis or cell sorting was conducted with a FACSARIA III Cell Sorter (BD Biosciences). Data were analyzed using FACSDiva software (BD Biosciences). The percentage of SatMs was calculated using the following formula: (ratio of SatM to Ly6C $^{-}$ F4/80 $^{-}$ Mac1 $^{+}$ cells in each sample/mean ratio of SatM to Ly6C $^{-}$ F4/80 $^{-}$ Mac1 $^{+}$ cells in vehicle control) \times 100%. After calculating the percentage for an individual mouse, the mean value was calculated.

2.5. Isolation and culture of SatMs

Mice were sacrificed by exsanguination under anesthesia with 3% isoflurane. Bone marrow cells were collected as previously described [11]. Collected cells were separated through the use of magnetic particles (IMag Ly-6 G/Ly-6C (Gr-1); BD Biosciences). SatMs were sorted from these cells using a FACSARIA III Cell Sorter. SatMs were seeded into 96-well plates at a density of 1×10^5 cells/well and cultured with RPMI-1640 containing 2 mM L-glutamine, 200 U/ml penicillin and 200 mg/ml streptomycin (Invitrogen) with or without 10 μ g/ml zymosan and AA6216 (3, 30, 300, and 3000 nM), apremilast (30, 300, and 3000

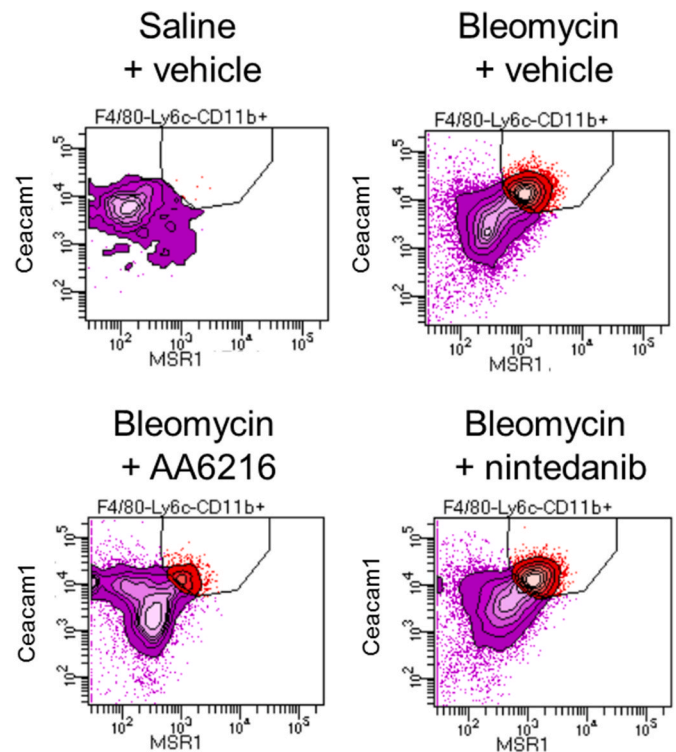


Fig. 1. Flow cytometric analyses of SatMs (Ceacam1 $^{+}$ Msr1 $^{+}$ Ly6C $^{-}$ F4/80 $^{-}$ Mac1 $^{+}$ monocytes) in BALF. Mice received a single dose of bleomycin or saline intratracheally at day 0, and vehicle, 10 mg/kg AA6216, or 100 mg/kg nintedanib were administered from day 0 to 8. BALF was collected on day 9.

nM), roflumilast (30, 300, and 3000 nM) or vehicle. After a 24 h culture period at 37 $^{\circ}$ C in 5% CO $_2$, the cultured media was harvested and stored at -80° C prior to quantification of TNF- α by ELISA. The inhibition (%) values were calculated using the following formula: (1-concentration of TNF- α in each sample/concentration of TNF- α vehicle control) \times 100%. The mean values were calculated from at least 3 independent

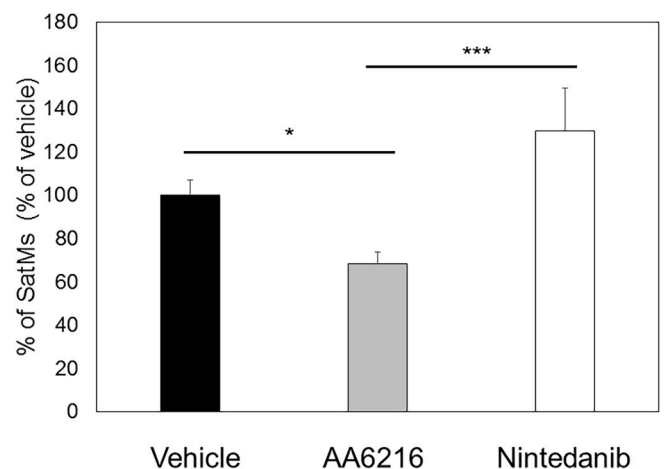


Fig. 2. The frequency of SatMs in BALF. Mice received a single dose of bleomycin or saline intratracheally at day 0, and vehicle, 10 mg/kg AA6216, or 100 mg/kg nintedanib were administered from day 0 to 9. BALF was collected on day 10. The ratio of SatMs to Ly6C $^{-}$ F4/80 $^{-}$ Mac1 $^{+}$ in the treatment groups is presented as a percentage of the ratio in vehicle-treated mice. The value of vehicle-treated mice was defined as 100%. Data are presented as mean \pm S.E.M. Vehicle: n = 20; AA6216: n = 20; nintedanib: n = 10. Statistical significance was determined by ANOVA with Tukey's posthoc comparisons test. *P < 0.05, ***P < 0.001.

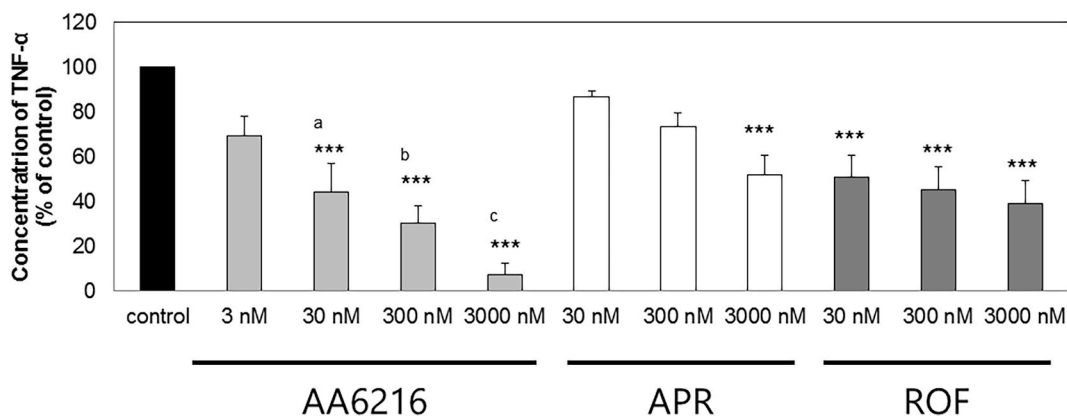


Fig. 3. AA6216 inhibits the production of TNF- α by SatMs. SatMs activated with zymosan were cultured with vehicle control, AA6216, apremilast (APR), or roflumilast (ROF) for 24 h. Concentrations of TNF- α were measured by ELISA, and the percentage of control values was calculated. The value of the control was defined as 100%. Data are presented as mean \pm S.E.M. and are compiled from 3 to 7 independent experiments. Statistical significance was determined by ANOVA with Tukey's posthoc comparisons test. *** P < 0.001 (vs control); ^a P < 0.05 (vs APR 30 nM); ^b P < 0.01 (vs APR 300 nM); ^c P < 0.01 (vs APR 3000 nM).

experiments.

2.6. Statistical analysis

Data were compared using ANOVA with Tukey's post hoc comparisons test, and P values less than 0.05 were considered statistically significant. Statistical analyses were performed using SAS 9.4 (SAS, version 9.4; SAS Institute, Inc., Cary, NC).

3. Results

3.1. AA6216 inhibited the accumulation of SatMs in the lung

PDE4 regulates the production of various cytokines and chemokines that contribute to immune cell migration [12–15]. Because SatMs accumulate in pro-fibrotic areas and cause fibrosis [7], we investigated the effect of AA6216 on the pulmonary accumulation of SatMs in the bleomycin-induced mouse model of lung fibrosis. Mice received bleomycin intratracheally at day 0 and either 10 mg/kg AA6216, 100 mg/kg nintedanib, or vehicle orally once daily from day 0 to 8. On day 9, we isolated the BALF and analyzed the ratio of SatMs (Ceacam1⁺ Msr1⁺ Ly6C⁻ F4/80⁻ Mac1⁺) to Ly6C⁻ F4/80⁻ Mac1⁺ cells by flow cytometry. Treatment with AA6216 reduced the frequency of SatMs in the lungs of bleomycin-induced mice compared to vehicle-treated and nintedanib-treated mice ($68.3 \pm 5.4\%$) (Figs. 1 and 2). In contrast, nintedanib did not affect the frequency of SatMs ($129.8 \pm 19.7\%$) (Figs. 1 and 2). These results suggest that AA6216 suppresses the pulmonary accumulation of SatMs.

3.2. AA6216 inhibited the production of TNF- α by SatMs

As SatMs activate fibroblasts through the overproduction of TNF- α [7], we next examined the inhibitory effects of AA6216 on the production of TNF- α by SatMs. We isolated SatMs from murine bone marrow cells and activated them with zymosan, and then cultured them for 24 h with AA6216, roflumilast or apremilast. Roflumilast and apremilast are also PDE4 inhibitors and are approved treatments for chronic obstructive pulmonary disease and psoriasis [16,17]. We then analyzed TNF- α levels in the culture supernatants by ELISA. All three PDE4 inhibitors, AA6216, roflumilast and apremilast, dose-dependently reduced the production of TNF- α by SatMs compared to the control (AA6216: 3 nM $69.2 \pm 9.0\%$, 30 nM $44.4 \pm 12.5\%$, 300 nM $30.3 \pm 7.8\%$, 3000 nM $7.3 \pm 4.9\%$, apremilast: 30 nM $86.7 \pm 2.6\%$, 300 nM $73.5 \pm 6.3\%$, 3000 nM $52.1 \pm 8.6\%$, roflumilast: 30 nM $51.0 \pm 9.5\%$, 300 nM $45.4 \pm 10.3\%$, 3000 nM $39.0 \pm 10.6\%$, respectively) (Fig. 3).

Importantly, the inhibitory effect of AA6216 was significantly stronger than that of apremilast at 30 nM (P < 0.05), 300 nM (P < 0.01), and 3000 nM (P < 0.01) doses. The inhibitory effect of AA6216 was also stronger than that of roflumilast at all tested doses, but the differences did not reach statistical significance.

4. Discussion

This study is the first to investigate the effects of PDE4 inhibitors on SatMs in a mouse model of lung fibrosis. We found that AA6216 suppressed the pulmonary accumulation of the fibrosis-specific macrophages SatMs, while the antifibrotic drug nintedanib did not. We also showed that AA6216 inhibited the production of TNF- α by SatMs.

Two macrophage subsets with distinct functions have been described. M1 macrophages mediate host defenses against a variety of bacteria, protozoa, and viruses, and are involved in anti-tumor immunity. In contrast, M2 macrophages are anti-inflammatory and regulate wound healing [18]. Recently, SatMs, fibrosis-specific macrophages that are not classified as M1 or M2, were identified in a bleomycin-induced mouse model of pulmonary fibrosis [7]. SatMs accumulate in the fibrotic area at the initiation of fibrosis and are critical for the development of lung fibrosis [7]. In our study, AA6216 significantly reduced the pulmonary accumulation of SatMs.

In contrast, nintedanib, which is used clinically as an antifibrotic agent, did not influence the frequency of SatMs. Nintedanib is an intracellular tyrosine kinase inhibitor that targets multiple tyrosine kinases, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) receptors. Although nintedanib is antifibrotic in bleomycin-induced murine pulmonary fibrosis models [19], PDGF, VEGF, and FGF may have less of an effect on SatMs. Thus, our data suggest that AA6216 exerts an antifibrotic effect by a mechanism distinct from that of nintedanib.

Pirfenidone is another drug that has been used clinically for the treatment of IPF [3], and it has been shown to be especially effective in combination with inhaled N-acetylcysteine [20]. Although the molecular target of pirfenidone has not yet been identified, this drug has been reported to inhibit polarization to M2 macrophages [21]. N-acetylcysteine has been shown to have antioxidant effects, to reduce oxidative damage to the mitochondria, and to reduce TNF- α release by alveolar macrophages [22]. Investigations into the effects of these drugs on SatMs may provide additional insight into their mechanisms and may therefore yield avenues for exploration of novel treatment strategies.

PDE4 inhibitors reduce the production of various cytokines and chemokines by immune cells [15], which may reduce mediators, such as CXCL12, required for SatMs accumulation within the lungs. A recent

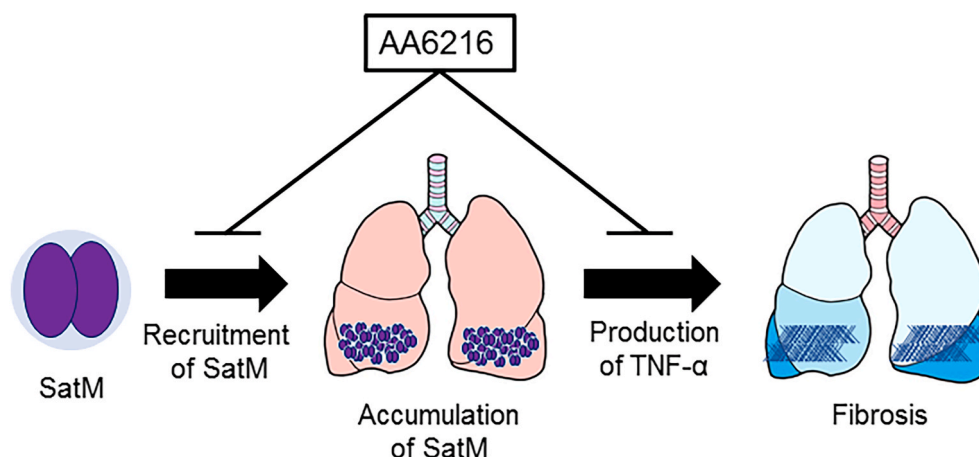


Fig. 4. The mechanism of action of AA6216 in regulating SatMs in lung fibrosis. AA6216 inhibits the recruitment of SatM to the fibrotic area and the production of TNF- α by SatM.

study showed that CXCL12 induced the recruitment of SatMs followed by the development of fibrosis [23]. In addition, the PDE4 inhibitor rolipram inhibits T-cell polarization and migration induced by CXCL12 [24]. Thus, AA6216 may inhibit the accumulation of SatM in the lungs by acting on pathways associated with CXCL12. Future studies are necessary to address this hypothesis.

The mechanisms by which SatMs contribute to the development of fibrosis are incompletely understood. They likely contribute to this pathology via multiple mechanisms. It has been established that SatMs activate fibroblasts via the production of relatively large amounts of TNF- α , but they do not produce TGF- β [7]. Therefore, it is speculated that SatM works at least in part through production of TNF- α [7]. It is important to note, then, that the PDE inhibitors tested here all inhibited the production of TNF- α at nanomolar concentrations (Fig. 3). AA6216, in particular, was a potent inhibitor of TNF- α production, and it was a significantly better inhibitor than apremilast.

Notably, AA6216 is a potential therapeutic agent for IPF not only through its SatM-related anti-fibrotic effects but also through its anti-inflammatory effects. For example, in previous studies, AA6216 was shown to inhibit TNF- α production by human PBMC [10,25] and by alveolar macrophages isolated from patients with IPF [10]. AA6216 was also shown to suppress skin inflammation in mouse dermatitis models [25]. These findings provide further support for the development of AA6216 as a tool for the treatment of IPF.

In regard to toxicology, it is important to consider inhibitor selectivity. AA6216 is highly selective for PDE4 [10], but it also inhibits PDE2A and PDE10A [10], which are reported to be expressed in the brain [26,27]. Fortunately, AA6216 has been shown to exhibit a strong tendency to migrate to the lung [10], which may reduce toxicity when administered in human patients. Accordingly, in mice, 10 mg/kg of AA6216 is well tolerated, in that it was not found to affect the general condition of mice in a previous study [10].

This study carries some limitations. First, in the bleomycin model, fibrosis is considered to be established at later time points, for example at day 21 [28]. However, our collection of SatM at day 9 is consistent with the initial report of SatM by Satoh et al., in which FACS analyses demonstrated that SatM levels increase in this model from day 7 to day 13 [7]. It will be important to evaluate the effect of AA6216 on SatM in the later fibrotic phase in further studies. Second, since no positive control compound has been found to have an inhibitory effect on SatM accumulation in the lungs, we chose to perform a comparison with nintedanib, which is used clinically as an anti-fibrotic agent. The effects of other PDE4 inhibitors, such as apremilast and roflumilast, as well as PDE1 inhibitors, which have recently been shown to inhibit fibrosis in animal models [29], on lung accumulation of SatM should also be investigated further.

A third potential limitation regards the dose of AA6216 used, as the effects were examined only at 10 mg/kg but not at other doses. Although high lung tissue concentrations and an anti-fibrotic effect were achieved at this dose in our previous study [10], the examination of dosage effects in future studies would provide valuable information regarding efficacy and safety. Similarly, future studies should investigate effects of treatment duration; here, treatment was performed for as long as possible prior to the harvesting of SatMs, but further studies should optimize the extent of treatment. Finally, only female mice were used, according to recommendations from a report of the American Thoracic Society ATS [30]. However, male mice develop more severe disease after bleomycin exposure than do females [31]. Therefore, identifying differences in effects of AA6216 on male and female mice represents an interesting avenue for future investigation.

5. Conclusions

Our studies suggest that AA6216 ameliorates lung fibrosis by inhibiting the recruitment of SatMs to the lung and reducing TNF- α production by SatMs. Therefore, AA6216 can suppress the pro-fibrotic activity of SatMs via two pathways (Fig. 4).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TM and YTA are employees of Meiji Seika Pharma Co., Ltd. YTO received research support from Meiji Seika Pharma Co., Ltd. related to this work.

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Abbreviations

BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
FGF	fibroblast growth factor
IPF	Idiopathic pulmonary fibrosis
PDE	phosphodiesterases
PDGF	platelet-derived growth factor

SatM	segregated-nucleus-containing atypical monocytes
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Ethics approval and consent to participate

All experiments were approved by the Animal Care and Use Committee of Kindai University, Faculty of Medicine (KAME-29-027 and KAME-31-034), and the Animal Care and Use Committee of Meiji Seika Pharma Pharmaceutical Research Center (2018-006-0, 2018-029-1, and 2019-009-0).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article. More details are available from the corresponding author upon reasonable request.

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Authors' contributions

TM and ON contributed to the concepts underlying the study. TM designed the study. TM and SK acquired the data. TM and ON contributed to the analysis or interpretation of the data. TM drafted the manuscript. ON, YTA, HS, TI, and YTO revised the content of the manuscript. All authors read and approved the final manuscript.

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